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The three protein components of anthrax toxin can be safely produced and separated from each other using newly developed conditions for growth of Bacillus anthracis and recovery of culture supernatant protein. Growth of the avirulent Sterne strain in a 50-liter fermentor yields up to 500 milligrams of the protective antigen component. Details of analysis showed that this protein has a small degree of heterogeneity caused by proteolytic nicking and by a second unknown mechanism that produces species differing slightly in charge,

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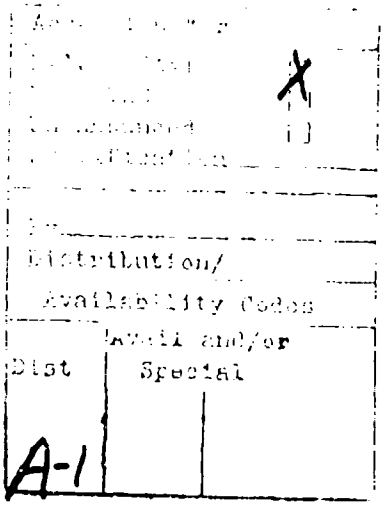

Anthrax toxin

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ANTHRAX TOXIN

By Stephen H. Leppla

Bacillus anthracis secretes three proteins which are collectively known as anthrax toxin.[1-3] The protective antigen (PA, 85 kd), lethal factor (LF, 83 kd), and edema factor (EF, 89 kd) proteins individually have no known toxic activities. Simultaneous injection of PA and LF causes death of rats, while PA and EF together produce edema in skin.[3] Thus, "anthrax toxin" is actually two toxins, each of which is like staphylococcal leukocidin[4,7] and botulinum C2 toxin[8] in having receptor binding and effector domains on separate proteins. The PA protein appears to play a dual role as the "B" moiety for two different "A" proteins. Binding studies have shown that PA must be present in order for EF to bind to cells.[9] EF is a calcium- and calmodulin-dependent adenylate cyclase which causes large increases in intracellular cAMP concentrations.[10] The mechanism of action of LF is unknown.

For B. anthracis to be fully virulent, it must produce two materials, the anthrax toxin and a polyglutamic acid capsule.[11] Recent work has shown that virulent strains possess two large plasmids. Plasmid pXO1 (114 megadaltons) codes for all three toxin components,[12-16] while pXO2 (60 megadaltons) codes for the polyglutamic acid capsule.[17-18] Methods are available to eliminate either or both plasmids. The genes for PA[13] and for LF[16] have been cloned, and the former has been sequenced.[19]

Of particular value in toxin research are strains possessing only pXO1 since these strains are at least 10^5-fold less virulent than strains that produce both toxin and capsule. The most

widely-used toxinogenic, noncapsulated strain is that designated Sterne, after its originator.[20] Suspensions of Sterne spores are employed as a vaccine for livestock and have also been used as a vaccine in man.[3]

The protective antigen protein was originally recognized by its ability to induce immunity to infection.[21] Extensive work led to the partial purification and eventual licensing of an aluminum hydroxide-adsorbed PA preparation as a human vaccine.[3,22] The work described below was initiated to find improved methods for vaccine production, and was later extended to include purification and characterization of the other two toxin components.

Safety Considerations

The anthrax toxins are not of extremely high potency,[23,24] and are not known to be absorbed from the digestive tract. Therefore, the normal precautions used in handling toxic chemicals provide adequate protection. An added degree of safety applies when the toxin components have been separated, since they have no known action individually.

The principal hazard is possible infection by the organism. If recognized early, infections are treated successfully with antibiotics, but symptoms are often nonspecific. At the author's laboratory, where both virulent and avirulent strains are under study, all bacteriological work is done in containment suites operated at the BL3 level. Personnel are immunized with the licensed human vaccine, purchased from the Michigan Department of Public Health. Virulent strains are grown in flasks in a shaking incubator, but not in fermentors, and swabs are taken at weekly intervals to detect any contamination of laboratory surfaces. Suspected accidental inoculation of organisms is sufficient basis for prophylactic administration of penicillin. Although the virulent strains present a much greater risk than Sterne-type strains, possible infection by the latter cannot be entirely discounted. Thus, it has recently been shown that relatively low doses of Sterne (10^3 spores) are lethal for certain inbred mouse lines.[25]

Therefore, the possibility that some persons may be unusually susceptible to infection by Sterne strains should be kept in mind, and large volume cultures, in particular, should be treated with appropriate care. For laboratories planning to grow only Sterne-type strains in volumes of less than 5 liters, the rigorous measures described above would be excessive. Some commercial producers of vaccine are believed to grow substantial volumes of Sterne with the usual concern for bacteriological safety, but do not immunize personnel. In those cases where the intent is to produce toxin rather than spores, cultural conditions should be chosen which prevent sporulation, thereby simplifying the problem of decontamination.

Bacterial Strain Selection

All natural isolates of B. anthracis appear to produce approximately the same amount of toxin (within 50%), and to produce all three toxin components. Thus, any pXO1+,pXO2- strain could be selected for toxin production. Investigators should use either well-characterized Sterne-type strains or verify that locally-derived, nonencapsulated strains lack pXO2. It is not correct to assume that every noncapsulated strain lacks pXO2, since some virulent strains can generate capsule-negative variants while retaining the pXO2 plasmid; these variants can revert to full virulence (T.M. Koehler, R.E. Ruhfel, B.D. Green, and C.B. Thorne, Am. Soc. Microbiol., Abstract H-178, 1986). Previous concern that true Sterne-type strains (pXO1+,pXO2-) might revert to virulence can now be dismissed, since the pXO2 plasmid carries essential capsule genes.

A readily available strain developed for protective antigen production[22] and used by the Michigan Department of Public Health for human vaccine preparation is V770-NP1-R (American Type Culture Collection, Accesssion No. 14185). This strain was selected as a nonproteolytic, noncapsulated mutant. While theoretically an advantage, the nonproteolytic characteristic does not seem to improve toxin yields when the medium described below

is used. Another readily available, attenuated strain, Pasteur vaccine No. 1, ATCC 4229, is not suitable for toxin production since it is pXO1-, pXO2+.[10]

The procedures described in this chapter were optimized for the Sterne strain, use of which is recommended. The Sterne strain is available from this and other laboratories, but is not currently deposited with the ATCC. Another suitable strain is a spontaneous, rifampicin-resistant Sterne mutant designated SRI-1, which has been found to produce 50-75% more toxin than Sterne. This strain appears defective in septum formation and grows in long filaments. This strain is not a good choice if a method generating high shear forces, such as tangential flow filtration, is used for removal of bacteria.

Strains are stored as either spore suspensions or frozen vegetative cells, and are revived on blood agar or other appropriate media. In this laboratory, aliquots of vegetative cells grown in RM medium (described below) and stored at -70° are thawed and spread on solid RM medium lacking bicarbonate and grown 24 h at 32° to prepare a fermentor inoculum. Growth at temperatures above 37° should be avoided since plasmid curing may occur.[12,14] If virulent B. anthracis are in use in the laboratory, aliquots of the fermentor inoculum should be grown in parallel on serum- or bicarbonate-containing medium and incubated in a CO_2 atmosphere to detect any capsulated contaminants.

Culture Medium and Growth Conditions

A number of investigators developed media that promote production of PA[22,26] or toxin.[2,27,28] Recent work at this laboratory developed "R" medium,[28] which was derived from one of the more successful semi-synthetic formulations[27] by replacement of casamino acids with an equivalent L-amino acid mixture (except that alanine was omitted to limit sporulation). Further media development work[32] was done to optimize yields of LF and EF in addition to PA and to facilitate product recovery. In these trials, yields of all three toxin components increased or decreased

in parallel. The modified medium developed through these trials was designated RM, and is described below.

The features of a medium which appear important for toxin production are: (1) inclusion of $NaHCO_3$, which aids pH control and also seems to have a chemical effect, perhaps by permeabilizing the bacteria;[29] (2) maintenance of pH > 7 (achieved here by $NaHCO_3$ and Tris), which serves to limit the action of proteolytic enzymes;[30] and (3) growth under essentially anaerobic conditions. Besides enhancing yields, anaerobic growth is advantageous because it places fewer demands on the fermentation equipment and decreases the potential for contamination of the laboratory.

RM medium contains the following ingredients at the indicated final concentrations (mg/liter), with all amino acids being of the L configuration: tryptophan (35), glycine (65), tyrosine (144), lysine hydrochloride (230), valine (173), leucine (230), isoleucine (170), threonine (120), methionine (73), aspartic acid (184), sodium glutamate (612), proline (43), histidine hydrochloride (55), arginine hydrochloride (125), phenylalanine (125), serine (235), NaCl (2,920), KCl (3,700), adenine sulfate (2.1), uracil (1.4), thiamine hydrochloride (1.0), cysteine (25), KH_2PO_4 (460), 2-amino-2(hydroxymethyl)-1,3-propandiol [Tris] (9,060), glucose (5000), $CaCl_2 \cdot 2H_2O$ (7.4), $MgSO_4 \cdot 7H_2O$ (9.8), $MnCl_2 \cdot H_2O$ (1.0), and $NaHCO_3$ (8000). The RM medium differs from R in containing NaCl, KCl, Tris, increased glucose (0.5% vs. 0.25%), decreased potassium phosphate (3.4 mM vs. 17.2 mM), and in substitution of cysteine for cystine. The latter substitution was done for convenience and is not known to enhance toxin yields. The medium is prepared with good quality distilled or deionized water. If extremely high quality water is used, addition of iron and trace metals may be required to support growth.

To prepare medium for a 50-liter fermentor culture, the first twenty-one ingredients in the above list (ending at thiamine) are added as solids to 40 liters of distilled water and sterilized in the fermentor vessel. The remaining eight ingredients are

individually dissolved in sterile water and sequentially pumped through a disposable capsule filter into the vessel, followed by water as needed to reach 50 liters. The medium in the vessel is then titrated to pH 8.0. For RM medium to be used in flasks, it is more convenient to group the ingredients into several stock solutions (for examples, see Haines et al.[27]); these are separately filter-sterilized and added to the flasks. Stocks of $NaHCO_3$ should be made fresh and filtered by pressure to avoid loss of CO_2, and media to which this stock has been added should be kept in tightly closed flasks. Failure of B. anthracis to grow in aged medium is usually due to alkaline conditions resulting from CO_2 loss.

One medium modification that consistently increases yields of all three toxin components by 50-100% is addition of horse serum to 3-5%.[24] Adding serum precludes purification (except by immunoadsorption -- see below), but the increased yields are useful when the goal is small-scale production of immunochemically or enzymatically active toxin. In cases where growth conditions are not optimal, adenylate cyclase activity may be detectable only in serum-supplemented cultures. Other proteins or putative protective agents and dialyzed horse serum seem to be less effective than horse serum. The basis of this effect is not known.

To grow a 50-liter fermentor culture, the bacteria on five RM agar plates are suspended in 25 ml RM medium, giving an $A_{540\ nm} = 8$; this suspension is added to the vessel. The culture is stirred at 150 rpm, regulated at 35°, controlled at pH 8.0 by addition of 1 M NaOH, and no aeration is used. If dissolved oxygen is measured, it is found that this falls to 0 once significant growth of the culture has occurred. The culture grows to $A_{540\ nm} = 2\text{-}2.5$ by 18-24 h, with logarithmic growth evident from the rate of NaOH consumption. Approximately 1-3 moles of NaOH are consumed. Fermentor cultures are harvested promptly after growth has ceased.

For growth in flasks, the containers are half-filled with medium, inoculated, tightly capped, and shaken at a speed just sufficient to maintain the bacteria in suspension. Cultures grown in flasks or in fermentors without pH control will show a fall in pH to 7.2-7.4. Toxin yields in such cultures are usually comparable to those in pH-controlled fermentor cultures.

Recovery of Toxin from Culture Supernatants

In cultures grown as described above, PA, LF, and EF are present at approximately 20, 5, and 1 μg/ml respectively, and collectively constitute more than half of the extracellular protein. Since separating the components from each other and from impurities is not difficult (see below), the principal challenge lies in recovering the dilute proteins from the culture supernatant. Immunoadsorbent chromatography has been used to recover and purify LF,[31] and could also be applied to PA and EF, but may not be economical if applied to unconcentrated supernatants. Ultrafiltration or batch adsorption to ion exchange resins can be used if appropriate equipment is available. Two alternate methods for toxin recovery will be described here, one that is currently in use with 50-liter fermentor cultures, and a second which may be more convenient for small-scale cultures.

Fermentor Cultures (>10 liters). When growth has ceased, 1,10-phenanthroline hydrochloride is added to give a final concentration of 0.05 mM; EDTA is added to 2 mM. These chelators inhibit a B. anthracis metalloprotease,[32] which is similar to that produced by B. cereus.[33] Phenylmethanesulfonyl fluoride is added to 0.1 mM, although it has not been proven that this enhances yields. Mercaptoethanol is added to 2 mM. The culture is chilled if the fermentor has this capability, and is pumped via a stainless steel coil immersed in an ice bath into a continuous-flow centrifuge (Sorvall TZ-28 rotor, or a comparable system that does not generate an aerosol). The TZ-28 rotor is operated at 12,000 rpm, and a flow rate of 400 ml/min is maintained with a peristaltic

pump. The supernatant is moved to a cold room, and all subsequent steps are performed at 4°. The supernatant is sterilized by filtration by using a Pellicon tangential flow ultrafiltration unit containing 10 ft^2 of Durapore 0.45-μm membrane (Millipore Corp.). Filtration rates exceeding 500 ml/min can be obtained in this unit. The membrane is treated with bovine serum albumin before its first use in order to prevent adsorptive losses. The membranes may be used to process a number of 50-liter cultures, provided that each use is followed b hing with 0.1 M NaOH. Tangential flow filtration systems ha eens on the retentate side have not been useful as a substi or the initial centrifugation, because the bacterial chains (average ten cells/chain for Sterne strain) plug the screens.

In the next step, the proteins are concentrated from the sterile supernatant by a "salting out" adsorption process.[34] Approximately 1 liter of cross-linked agarose beads (Sepharose CL-4B, Pharmacia, or a similar resin) is added to the supernatant, followed by the slow addition of 25 kg $NH_4(SO_4)_2$. The suspension is stirred gently until the salt dissolves (2-3 h) and then the agarose beads are allowed to settle. Every effort is made to reach this point on the same day the culture is harvested. If successful, it is convenient to let the resin settle overnight. The supernatant is pumped off, passing it through a porous plastic funnel (Bel-Art Plastics) if necessary to collect any resin that has not settled. The agarose is then placed in a 14-cm diameter column and eluted at 25 ml/min with 2 liters of 50 mM Tris, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 8.0. The fractions containing >95% of the protein are pooled and precipitated by slow addition of solid $NH_4(SO_4)_2$ to 75% saturation. After 2-24 h the precipitated toxin is collected by centrifugation, redissolved in 100 ml 10 mM Tris, 0.05 mM 1,10-phenanthroline, 2 mM 2-mercaptoethanol, pH 8.0, and dialyzed against the same buffer. At the author's facility, it was found convenient and feasible at this stage to filter-sterilize the toxin and remove it from the BL3 laboratory. Subsequent purifications are done at the BL1 contain-

ment level. Successful preparations contain 2-4 g protein, as determined by UV absorption, assuming $E_{1\%}$(280 nm, 1cm)=10. The phenanthroline in the sample absorbs at 280 nm, but this does not interfere if dialysate is used to blank the spectrophotometer.

Small Scale Cultures (0-10 liters). For these cultures, it may be more convenient to use a protocol employing adsorption to hydroxyapatite. LF and EF can be adsorbed from undiluted culture supernatants due to the low phosphate concentration in RM medium (3.4 mM, 20% of that in R medium). PA will not be completely adsorbed under the same conditions, but recovery of PA can be made nearly complete by addition of polyethylene glycol. Cultures grown in flasks or small fermentors are centrifuged to remove bacteria, chilled, adjusted to pH 7.0 with acetic acid, and protease inhibitors are added as described for large cultures, with the exception that EDTA is omitted[35] and 1,10-phenanthroline is increased to 0.2 mM. To each liter of supernatant, 5 g of hydroxyapatite (Fast Flow type, Calbiochem) is added and, if high recovery of PA is desired, 100 g of polyethylene glycol 8000 (PEG) is also added. The mixture is gently agitated at 5° until the PEG is dissolved and then an additional 1-3 h, avoiding use of a magnetic stir bar. The hydroxyapatite is transferred to a small column or filter funnel and washed with 10 mM Tris, pH 7.5. If PEG is not used, the lower viscosity makes it feasible to pass the culture supernatant directly through a column of hydroxyapatite. The toxin is eluted with 0.66 M potassium phosphate, pH 7.0. The eluate is supplemented with EDTA to 0.01 M, and is dialyzed against 10 mM Tris, 2 mM EDTA, pH 8.0, or other suitable buffer, depending on the intended use of the preparation. Toxin prepared in this way has not been as fully characterized as that made in fermentors. The reported presence of aldehydes and peroxides in PEG and related polyether detergents[36] raises concerns that subtle chemical damage may exist in proteins made using PEG.

Chromatographic Separation of Toxin Components

A number of chromatography methods are available to separate the toxin components. Details are given here for processing the amount of toxin obtained from a 50-liter fermentor, by using sequential chromatography on hydroxyapatite and DEAE-Sepharose. This order is preferred over the inverse, since EF was found to elute in several distinct peaks when crude toxin was run on DEAE, a result that may reflect a weak interaction of EF with PA. In the protocols described below, all operations are performed at 4°, and dialysis times should not exceed 16 h, except for the final product.

The dialyzed crude toxin (2-4 g) is pumped onto a 2.6x38-cm (200 ml) column of hydroxyapatite (Fast Flow type, Calbiochem) previously equilibrated to 0.005 M potassium phosphate, 0.1 M NaCl, 0.05 mM 1,10-phenanthroline, 2 mM 2-mercaptoethanol, pH 7.0 (Buffer A). The column is washed with at least 150 ml Buffer A at 50 ml/h and then eluted with a gradient of 500 ml each of 0 and 0.5 M potassium phosphate, pH 7.0, both in Buffer A. Fractions of 10 ml are collected in tubes containing 0.1 ml 100 mM EDTA. The components elute in the order PA > LF > EF, and each is evident as a peak of UV-absorbing material. The EF peak is small compared to the others, but is easily identified because it is the last significant peak. PA and LF are concentrated from pooled fractions by ammonium sulfate precipitation at 75% saturation and dialysis against 0.01 M Tris, 0.025 M NaCl, 0.05 mM 1,10-phenanthroline, 2 mM 2-mercaptoethanol, 1% glycerol, pH 8.0 (Buffer B). The EF is concentrated by ultrafiltration to about 25 ml and is dialyzed against buffer B.

Final purification of each component is achieved by chromatography on DEAE Sepharose CL-4B (Pharmacia), with a column containing about 1 ml resin per 5-10 mg input protein. As an example, 700 mg PA is purified on a 1.6x50-cm (100 ml) column, with a gradient of 750 ml each of 0 and 0.25 M NaCl in buffer B. For LF and EF, the high salt buffers should contain 0.40 and 0.25 M NaCl respectively. The protein in the pooled fractions is

dialyzed against 5 mM HEPES, 50 mM NaCl, pH 7.5, filter-sterilized with low protein binding filters (Millex-GV, Millipore), quick frozen in small aliquots, and stored at -70°.

Successful purifications yield 400 mg PA, 75 mg LF, and 20 mg EF. The LF and EF proteins appear as single species on SDS gels and analytical ion exchange HPLC (monoQ resin, Pharmacia); these components appear homogeneous. In contrast, two types of heterogeneity in PA have been observed. A variable fraction (usually < 10%) of the PA contains a cryptic polypeptide cleavage approximately 330 residues from the N-terminus. Electrophoresis under denaturing conditions reveals fragments of 37 and 47 kd. The site which is cleaved by the endogenous Bacillus protease is also highly susceptible to specific cleavage by other proteases, including chymotrypsin.[37] The two large peptide fragments produced by intentional cleavage at this site will be useful in structure-function analyses of PA. The other type of heterogeneity in PA consists of differences in net charge. During the final chromatography on DEAE, most preparations show one or two partially-separated, trailing species. These species are indistinguishable on SDS gels, but migrate as 2-4 evenly spaced bands after isoelectric focusing or non-denaturing, continuous slab gel electrophoresis. Careful selection of fractions, or chromatography on MonoQ with shallow gradients (Tris buffer, pH 8.0, 0.25-0.35 M NaCl) yields distinct, homogeneous preparations. The species with the least negative charge (eluting first from DEAE and monoQ) has been shown to have significantly greater potency (with LF) in the macrophage lysis assay described below. The nature of the alteration that introduces additional negative charge and decreases potency is not known. A similar type of heterogeneity has been observed in Pseudomonas exotoxin A.[38]

Toxin Assay

The anthrax toxins can be detected and quantitated according to their immunochemical, enzymatic, or toxic activity, or by direct chemical measurement.

Chemical Assays. For laboratories beginning purification of the toxin and not yet having component-specific antisera, it may be most convenient to perform direct chemical measurement of the toxin components by rapid electrophoresis or chromatography methods. The large size (83-89 kd) and the high concentrations of the proteins in RM medium cultures makes it relatively simple to locate the bands or peaks of PA and LF. While the three toxin components are similar in size, they can be distinguished on 8 or 10% polyacrylamide SDS slab gels[39] if small amounts of protein (0.1 μg per band) are loaded. The three proteins are well separated during ion-exchange high performance liquid chromatography (MonoQ), eluting in the order PA > EF > LF when the column is developed with a NaCl gradient at pH 8.

Immunochemical Assays. Antisera to the toxin components can be elicited using standard techniques.[40] Purified PA and LF give rise to high-titer sera, while EF seems less immunogenic and has not yielded sera useful in gel diffusion systems. Antisera to PA may be obtained by immunization with the licensed human vaccine (Michigan Department of Public Health), which contains principally this toxin component. Immunization with the live spore veterinary vaccine (Anvax, Jensen-Salsbery Laboratories, St. Louis, Mo.) induces antibodies to all three toxin components. Goat and rabbit sera have been used successfully in gel diffusion,[26] rocket immunoelectrophoresis,[24] immunoblots,[13] and ELISA.[13,16] For routine detection of PA and LF in column eluates, gel diffusion in agar is preferred because antigens can be detected over a wide range of concentrations. For quantitative measurement of PA, radial immunodiffusion employing specific goat antiserum has been most useful. Monoclonal antibodies to all three toxin

components have been developed[41] and can be expected to replace the polyclonal sera in some assays. Due to the low potency of the available sera, EF is usually assayed enzymatically (see below).

Adenylate cyclase assay. Of the three toxin proteins, only EF is known at this time to have enzymatic activity. The EF adenylate cyclase has enzymatic properties resembling those of the Bordetella pertussis adenylate cyclase.[42,43,44] An exception is that in all preparations of EF, the enzyme activity has been totally dependent on addition of calmodulin; no calmodulin-independent forms like those of the B. pertussis enzyme have been detected. Because EF has high enzymatic activity (V_{max} = 1.2 millimoles cAMP/min/mg[P]), any of the methods developed for the more difficult task of measuring eukaryotic adenylate cyclases may be used. The assay described below is modified from that of Solomon[45] by dilution of ^{32}P-ATP to lower specific activity, omission of a phosphodiester inhibitor, omission of the ^{3}H-cAMP added to determine chromatographic recoveries, and optional use of ^{3}H- or ^{14}C-ATP as a substitute for ^{32}P-ATP. This method has a higher sensitivity than is needed, but is preferred because the Dowex and alumina columns are reusable. Manganese ion is used in order to make the enzyme activity independent of calcium ion concentration. Samples and buffers should be chosen so as to exclude phosphate, which is a strong inhibitor (K_i about 0.01 mM).

Reagents:

Assay buffer(5X): pH 7.5

100 mM HEPES, 25 mM $MnCl_2$, 2.5 mM $CaCl_2$,
2.5 mM EDTA, 0.25 mM cAMP, 2.5 mM dithiothreitol
0.5 mg/ml bovine serum albumin (calmodulin-free[43])

^{32}P-ATP(10X): 0.02 mCi/ml, 5mM ATP

If assays are done infrequently, it may be preferred to use ^{3}H- or ^{14}C-ATP. These provide adequate sensitivity and long halflife, but require counting in scintillation fluid.

Calmodulin, bovine (10X): 0.05 mg/ml

Protocol: The reaction mixtures contain 0.020 ml assay buffer(5X), 0.010 ml calmodulin (10X), EF sample (2-10 ng) and water totalling 0.080 ml, and 0.010 ml ^{32}P-ATP(10X). Mixtures are preincubated 20 min before addition of ATP, to allow association of EF, calmodulin, and calcium. Controls should include reactions (1) with excess EGTA to remove calcium, (2) omitting calmodulin, and (3) with excess EF (0.01 mg/ml) to cause complete ATP conversion. The latter allows calculation of chromatographic recovery. After 60 min at 23°, 0.10 ml of stopping solution (2% sodium lauryl sulfate, 45 mM ATP, 1.3 mM cAMP) is added, the samples are heated 5 min at 95-100°, and 1.0 ml of water is added. The samples are poured into disposable plastic chromatography columns having integral 10 ml reservoirs (Bio-Rad) and packed with 1.0 ml Dowex AG 50W-X4 (Bio-Rad), followed by two portions of 3.0 ml water, taking care to let the fluid run completely into the resin after each addition. The Dowex columns are then placed over columns of the same design containing 1.0 ml neutral alumina WN-3 (Sigma), and 7 ml water is added to elute the cAMP from the Dowex and transfer it to the alumina. After the 7.0 ml has drained through the alumina columns, these are placed above scintillation vials, and the cAMP is eluted with 7.0 ml 0.1 M imidazole hydrochloride,

pH 7.0. The ^{32}P-cAMP is measured by Cerenkov counting. If ^{3}H- or ^{14}C-ATP is used as substrate, an aliquot is transferred to another vial containing an aqueous scintillation fluid. When the Dowex and alumina columns are first set up, ATP and cAMP standards should be run and fractions collected to verify elution positions. The results obtained may show that the ^{3}H- or ^{14}C-cAMP can be collected in a smaller volume so as to facilitate scintillation counting.

Toxicity assays. Anthrax toxin was originally defined as an agent causing edema in skin (now known to reflect the action of PA with EF), and subsequently was found to contain a material lethal for guinea pigs and rats (LF, when combined with PA). The skin edema assay is laborious and rather variable, and has been replaced in this laboratory by the Chinese hamster ovary (CHO) cell elongation assay.[9] The rat lethality assay remains useful as a quick and accurate measure of potency for PA and LF.[24] These toxicity assays can be performed directly on B. anthracis culture supernatants, since these do not contain other substances having measurable toxicity. All bioassays of unfractionated anthrax toxin must take into account the competitive action of LF and EF;[9,10,24] these components will inhibit the toxicity of the heterologous component unless diluted to concentrations below 0.1 μg/ml.

Rat lethality assays require use of male Fischer 344 rats weighing 250-300 g. Toxin samples or mixtures of components are diluted in buffer containing 0.1% bovine serum albumin. Measured volumes of 1.0-2.0 ml are injected into the dorsal penile vein. Times to death (40-200 min) are recorded and related to a standard curve.[24,27] If good injections are made, duplicate animals will have times to death differing by 2-5 min. Either PA (5-25 μg) or LF (2-10 μg) may be accurately measured if the complementary protein is injected in excess (> 60 μg). With PA and LF at 5:1 (wt/wt), the minimum lethal dose is 3 μg PA and 0.6 μg LF.[24] In

this assay, injection of 2.0 ml of a successful RM culture results in a time to death of 50-60 min.

Although most cultured cells do not show acute effects when exposed to anthrax toxin, several cell systems do provide useful assays for the toxin. Most fibroblast cell lines treated with PA and EF show a rise in concentration of intracellular cAMP,[10] which can be measured by radioimmunoassay[46] after extraction into dilute acid. CHO cells also undergo a morphological change, which forms the basis of a convenient and sensitive assay for PA and EF, but one which is difficult to quantitate. The combination of PA and LF does not kill most cells. The exception is mouse and rat macrophages, which lyse after a 2 h treatment with PA and LF at 0.1 ug/ml each.[47] This combination also slows the growth of certain cells, such as BHK-21, but this effect can be demonstrated only if cells are seeded at very low density, exposed to toxin, and cultured for several days.

Summary and Future Developments

Study of anthrax toxin has been essential in improving our understanding of the virulence of B. anthracis and in design of improved vaccines. In addition, study of the anthrax toxins may show them to be useful tools in cell biology. The adenylate cyclase toxin (PA+EF) has already been employed to study the effect of increased intracellular concentrations of cAMP. For this purpose it may be preferred over cholera toxin because the effect is rapidly reversed after toxin removal.[10] Expanded use of the anthrax toxins as pharmacological tools may occur in the future when the molecular basis of LF action is determined and when methods for toxin production are developed which do not require growth of B. anthracis. In the latter regard, recent work at this Institute has succeeded in cloning the PA gene into B. subtilis on plasmid pUB110.[48] Even without medium optimization, PA production and secretion by this strain equalled that by Sterne. It can be expected that use of protease-deficient B. subtilis hosts,[49] optimized medium, and alteration of the DNA

sequences controlling expression will combine to further increase yields.

REFERENCES and FOOTNOTES

1. H. Smith and J.Keppie, Nature (London) 173, 689 (1954).

2. C. B. Thorne, D. M. Molnar, and R. E. Strange, J. Bacteriol. 79, 450 (1960).

3. P. Hambleton, J. A. Carman, and J. Melling, Vaccine 2, 125 (1984).

4. S. H. Leppla, in "Microbiology-1985" (L. Lieve, ed.), p. 63. American Society for Microbiology, Washington, D.C. (1985).

5. J. L. Stanley and H. Smith, J. Gen. Microbiol. 26, 49 (1961).

6. A. M. Woodin, in "Microbial Toxins, Vol. III" (T. C. Montie, S. Kadis, and S. J. Ajl, eds.), p. 327. Academic Press, New York, (1970).

7. M. Noda, T. Hirayama, F. Matsuda, and I. Kato, Infect. Immun. 50, 142 (1985).

8. M. Iwasaki, I. Ohishi, and G. Sakaguchi, Infect. Immun. 29, 390 (1980).

9. S. H. Leppla, in "Advances in Cyclic Nucleotide and Protein Phosphorylation Research," Vol. 17 (P. Greengard et al, eds.), p. 189. Raven Press, New York (1984).

10. S. H. Leppla, Proc. Natl. Acad. Sci. U.S.A. 79, 3162 (1982).

11. C. B. Thorne, Ann. N. Y. Acad. Sci. 88, 1024 (1960).

12. P. Mikesell, B. E. Ivins, J. D. Ristroph, and T. M. Dreier, Infect. Immun. 39, 371 (1983).

13. M. H. Vodkin and S. H. Leppla, Cell **34**, 693 (1983).

14. C. B. Thorne, in "Microbiology-1985" (L. Lieve, ed.), p. 56. American Society for Microbiology, Washington, D.C. (1985).

15. EF gene was shown to be on pXO1 by demonstrating adenylate cyclase activity in B. cereus pXO1+ transcipients; C. B. Thorne and S. H. Leppla, unpublished, 1985.

16. D. L. Robertson and S. H. Leppla, Gene in press, 1986.

17. I. Uchida, T. Sekizaki, K. Hashimoto, and N. Terakado, J. Gen. Microbiol. **131**, 363 (1985).

18. B. D. Green, L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins, Infect. Immun. **49**, 291 (1985).

19. S. L. Welkos, in preparation, 1986.

20. M. Sterne, Onderstepoort J. Vet. Sci. Anim. Ind. 8, 279 (1937).

21. G. P. Gladstone, Br. J. Exp. Pathol. 27, 394 (1946).

22. M. Puziss, L. C. Manning, J. W. Lynch, E. Barclay, I. Abelow, and G. G. Wright, Appl. Microbiol. **11**, 330 (1963).

23. D. M. Gill, Microbiol. Rev **46**, 86 (1982).

24. J. W. Ezzell, B. E. Ivins, and S. H. Leppla, Infect. Immun. **45**, 761 (1984).

25. S. L. Welkos, T. J. Keener, and P. H. Gibbs, Infect. Immun. **51**, 795 (1986).

26. C. B. Thorne and F. C. Belton, J. Gen. Microbiol. 17,505 (1957).

27. B. W. Haines, F. Klein, and R. E. Lincoln, J. Bacteriol. 89, 74 (1965).

28. J. D. Ristroph and B. E. Ivins, Infect. Immun. 39, 483 (1983).

29. M. Puziss and M. B. Howard, J. Bacteriol. 85, 237 (1963).

30. R. E. Strange and C. B. Thorne, J. Bacteriol. 76, 192 (1958).

31. E. J. Machuga, G. J. Calton, and J. W. Burnett, Toxicon 24, 187 (1986).

32. S. H. Leppla, unpublished studies, 1984.

33. B. Holmquist, Biochemistry 16, 4591 (1977). See also this series, Vol XIX, p. 569.

34. F. von der Haar, Biochem. Biophys. Res. Commun. 70, 1009 (1976). See also R K. Scopes, "Protein Purification: Principals and Practice", p. 141, Springer-Verlag, New York, Heidelberg, and Berlin, 1982.

35. EDTA is omitted because it would extract the calcium from hydroxyapatite ($CaSO_4$), and might both dissolve the adsorbant and prevent protein binding. Phenanthroline does not dissolve hydroxyapatite, even when present at 20 mM.

36. W. J. Ray, Jr. and J. M. Puvathingal, Anal. Biochem. 146, 307 (1985). See also this series, Vol 52, p. 145, and Vol 56, p. 742.

37. S. H. Leppla, in preparation, 1986.

38. L. T. Callahan III, D. Martinez, S. Marburg, R. L. Tolman, and D. R. Galloway, Infect. Immun. 43, 1019 (1984).

39. U. K. Laemmli, Nature 227, 680 (1970).

40. B. A. L. Hurn and S. M. Chantler, this series, Vol 70, p. 104, 1980.

41. S. F. Little, in preparation, 1986.

42. E. Hewlett and J. Wolff, J. Bacteriol. 127, 890 (1976).

43. J. Wolff, G. H. Cook, A. R. Goldhammer, and S. A. Berkowitz, Proc. Natl. Acad. Sci. U.S.A. 77, 3841 (1980).

44. D. L. Confer and J. W. Eaton, Science 217, 948 (1982).

45. Y. Salomon, in "Advances in Cyclic Nucleotide Research," Vol. 10 (G. Brooker, P. Greengard, G. A. Robison, eds.), p. 35, Raven Press, New York, 1979.

46. G. Brooker, J. F. Harper, W. L. Terasaki, and R. D. Moylan, in "Advances in Cyclic Nucleotide Research," Vol. 10 (G. Brooker, P. Greengard, G. A. Robison, eds.), p. 1, Raven Press, New York, 1979.

47. A. M. Friedlander, J. Biol. Chem., in press, 1986.

48. B. E. Ivins and S. L. Welkos, in preparation, 1986.

49. F. Kawamura and R. H. Doi, J. Bacteriol. 160, 442 (1986).